Method of obtaining dendritic cells, dendritic cells thus obtained and uses thereof for clinical purposes

The present invention relates to the field of immunotherapy and more particularly to that of dendritic cells and the use thereof as an immunotherapeutic agent.

Dendritic cells (DC) play a key role in the initiation of primary immune response, and pilot clinical studies have demonstrated their capacity to induce efficient antitumor immunity.

Dendritic cells, which are present in the skin (Langerhans' cells), the mucosa, the peripheral blood and the bone marrow, are the most potent antigen presenting cells (APC) in the immune system. They are characterized by a unique morphology and a specific surface phenotype.

In particular, they express the CD83 antigen and are capable of expressing large amounts of MHC classes I and II and initiating mixed leukocyte reactions (MLR). On the other hand, they are devoid of certain myeloid markers, especially the CD14 marker.

Given their specific properties, these cells have been proposed as essential elements in cell therapies which require the presentation of antigens to T lymphocytes.

Dendritic cells (DC) have a specific mode of differentiation which comprises two major stages, namely the immature stage and the mature stage, according to a set of phenotypic and functional characteristics (1,2). Immature DC obtained *in vitro* from monocytes by culture with a granulocyte-macrophage colony stimulating factor (GM-CSF) and an interleukin that blocks differentiation towards the macrophagic pathway (IL-4 or IL-13) are analogous to peripheral tissue DC, i.e. Langerhans' cells and interstitial dendritic cells. These immature DC are capable of capturing antigens with great efficiency using specialized receptors such as the immunoglobulin Fc receptors (FcR) (3,4), the mannose receptor (MR) (5) and the phagocytic receptors, particularly CD36 and the $\alpha v\beta 5$ integrin (6). They can thus internalize proteins, whole cell lyzates, RNA and apoptotic cells. On the other hand, they only express low levels of co-stimulatory molecules necessary for T lymphocyte activation.

When exposed to maturation signals given mainly by antigens, inflammatory cytokines or bacterial products, DC lose their phagocytic and endocytic capacities (5,6) but increase the expression of major histocompatibility

complex (MHC) class I and MHC class II and the expression of CD80 and CD86 and become very potent antigen presenting cells (APC). The transition from the immature stage to the mature stage is associated with expression of the chemokine receptors. Mature DC have a reduced expression of CCR1 and CCR5, which are the receptors for inflammatory chemokines, the macrophage inflammatory proteins MIP-1α and MIP-1β and RANTES, and they concomitantly have an increased expression of CCR7, which is the receptor for the E1B ligand (ELC)/MIP-3β expressed constitutively in secondary lymphoid organs (7-9). These changes in the expression of the chemokine receptors are important for the *in vivo* circulation of DC. Immature DC are recruited by the inflammatory chemokines in the antigen entry sites. After activation by antigens and inflammatory stimuli, they lose the CCR1 and CCR5 receptors and acquire the expression of CCR7. Mature DC can then enter the lymphatic vessels and migrate towards the afferent lymphatic nodes, where they present antigen-derived epitopes for the naive lymphocytes and the memory lymphocytes present in these nodes.

Thus it has already been proposed to use them as vectors for antitumor vaccinations (10). Nestle et al. have recently shown that the intralymphatic injection of immature DC activated with tumoral peptides or tumor cell lyzates could elicit an antimelanoma immune response (11).

The use of dendritic cells for immunotherapeutic purposes requires many times several million cells. Furthermore, these cells must be capable of circulating in the human body selectively towards the nodes for the treatment to be effective. It is also important to have cells which are irreversibly engaged in the dendritic differentiation pathway, i.e. mature cells which are not likely to transform into macrophages in the organism.

Several studies concerning the modulation of the chemokine receptors have been performed with DC obtained by culture in a medium containing fetal calf serum (FCS). Now, xenogenic antigens can be immunodominant and can hamper the development of specific antitumor immunity.

Various research groups have therefore focused on the production of DC derived from monocytes in FCS-free media by using media supplemented with 1 to 10% of autologous plasma (12-17), autologous serum (18) or pooled human AB sera (13,19-22,31). However, even autologous serum may present a problem since it contains numerous proteins, especially antibodies (23), which can modify the pathway for binding and intracellular modification of the antigens. In addition,

some tumoral antigens of the MUC-1 type in several cancers, or the monoclonal immunoglobulin in multiple myeloma, are present in the serum at high and variable levels, which may affect a reproducible presentation by the DC.

For all these reasons, methods of obtaining DC capable of activating T lymphocytes in serum-free media have been proposed (24-26).

International patent applications WO 98/23728, WO 98/06823 and WO 98/06826 also describe methods of obtaining dendritic cells in serum-free media. International patent application WO 98/06826 describes, inter alia, the use of a serum-free medium, namely X-VIVO 15 medium supplemented with 1% of human albumin (HA). Said patent application states that the use of 1% of HA does not significantly improve the growth of the cells, their phenotype or their stimulatory capacity. Furthermore, the expression of CD86 is increased after 14 days of culture in said medium.

It has now been found, surprisingly, that it is possible to obtain large amounts of dendritic cells useful in immunotherapy by the culture of specific mononuclear cells in a serum-free medium appropriately supplemented with human albumin, in the presence of a granulocyte-macrophage colony stimulating factor (GM-CSF) and a cytokine, particularly interleukin-4 (IL-4) or interleukin-13 (IL-13), and then in the presence of at least one inflammatory mediator, for example tumor necrosis factor alpha (TNF-α).

Thus the method of the invention consists in:

1) cultivating for 4 to 6 days, preferably 5 days, mononuclear cells derived from cytapheresis after mobilization, in a serum-free medium supplemented with human albumin, in the presence of a granulocyte-macrophage colony stimulating factory (GM-CSF) and an interleukin (IL) that blocks differentiation towards the macrophagic pathway;

2) adding TNF-α and optionally an inflammatory mediator to the culture medium and continuing the culture for about a further 1 to 4 days, preferably 2 days; and

3) recovering the dendritic cells formed.

Advantageously, fresh medium containing GM-CSF and an interleukin can be added to the culture medium on days 2 and 4.

In one mode of carrying out the method of the invention, it is possible to use prostaglandin E2 (PGE2) together with TNF-α.

"Serum-free culture medium" is understood as meaning any culture

medium commonly used for the culture of cells for clinical purposes and containing the nutrients essential for the growth of hemopoietic cells, especially a source of carbon, nitrogen and transferrin.

These media are free of human serum and animal serum.

Examples of serum-free culture media appropriate for the purposes of the invention are described e.g. in WO 95/00632 and US 5 405 772.

Specific examples of such media are the X-VIVO 10 and X-VIVO 15 media marketed by Biowhittaker, Walkersville, MD, USA.

X-VIVO 15 medium is particularly preferred for carrying out the invention.

The culture medium must be supplemented with human albumin at a rate of 1 to 2% (weight/volume), preferably 2%.

"Mononuclear cells" are understood as meaning mononuclear cells (MNC) derived from the peripheral blood of normal subjects or of patients presenting with a cancer or any other disease involving the immune system, such as infectious, viral or parasitic diseases, for example AIDS, or immunodeficiency disorders, for example rheumatoid arthritis, lupus, etc.

The mononuclear cells (MNC) used as the starting material in the method according to the invention are obtained by cytapheresis after mobilization by chemotherapy and/or with at least one cell growth factor.

Thus the mononuclear cells used in the method of the invention are derived either from normal subjects or from cancer patients who have undergone a chemotherapy, namely a specific treatment with a chemotherapeutic agent and optionally a cell growth factor, or from patients presenting with an infectious, viral or parasitic disease who have been treated with a cell growth factor, such as cytokines, including hemopoietic growth factors.

The following may be mentioned as examples of growth factor which can be used to mobilize the mononuclear cells:

- granulocyte colony stimulating factors (G-CSF) such as the products known under the trade names "filgrastim NEUPOGEN" and "Lenograstim GRANOCYTE" from Amgen-Roche and Rhône-Poulenc/Chugai respectively; and

- granulocyte-macrophage colony stimulating factors (GM-CSF) such as the products known under the trade name LEUCOMAX from Schering Plough or the stem cell growth factor (SCF) from Amgen.

The mobilized mononuclear cells used according to the invention include especially monocytes, lymphocytes and hemopoietic stem cells.

Mobilization by chemotherapy is effected with the chemotherapeutic agent appropriate to the type of cancer presented by the patient donating the cells to be used in the method of the invention. Any chemotherapeutic agent can be used, an example being cyclophosphamide.

The amounts of GM-CSF, interleukin, TNF-α and PGE2 to be used in the method of the invention are those normally used for cell cultures.

It is specified that GM-CSF can be used at a rate of 1 ng/ml to 1000 ng/ml, preferably 50 to 500 ng/ml and advantageously 100 ng/ml of medium.

Interleukin is generally used in amounts ranging from 1 ng/ml to 1000 ng/ml, preferably 10 to 50 ng/ml and advantageously 25 ng/ml of medium.

Also, TNF-α can be used at a rate of 1 ng/ml to 1000 ng/ml and PGE2 at a rate of 10 ng/ml to 10 μg/ml and advantageously 20 ng/ml to 1 μg/ml.

The precursor cells of dendritic cells are cultured in plastic containers commonly used in this field, such as cell culture flasks or bags, which allow adherence of the cells.

The culture is advantageously performed in incubators under normal cell culture conditions (sterility; CO_2 about 5%; humidity about 95% and temperature about 37°C).

According to another feature, the invention relates to dendritic cells that are $\alpha v\beta_3^-$, $\alpha v\beta_5^+$, $CCR5^-$ and $CCR7^+$, i.e. are devoid of $\alpha v\beta_3^-$ and CCR5 receptors and carry $\alpha v\beta_5$ and CCR7 receptors.

These dendritic cells, which can be obtained by the method defined above, are irreversible mature cells. They can be used as immunotherapeutic agents in all cell therapies, for example the treatment of cancers or infectious, viral or parasitic diseases.

Thus the invention further relates to the use of $\alpha v\beta_3^-$, $\alpha v\beta_5^+$, $CCR5^-$ and $CCR7^+$ dendritic cells for the production of an immunotherapeutic agent useful for the treatment of any disease involving the immune system.

In fact, with the dendritic cells according to the invention, it is possible to reduce the internalization and presentation of unidentified xenogenic, allogenic or autologous proteins, thereby limiting the immune responses which are not specific to the tumoral antigens.

The DC according to the invention are capable of capturing tumoral antigens *in vivo* either by endocytosis of the proteins or by phagocytosis of the apoptotic cells.

These DC are capable of migrating towards the lymphatic nodes so as to present the peptides derived from antigens directed against the T lymphocytes. They are also capable of producing interleukin-12, favoring the differentiation of naive CD8+ cells into cytotoxic T lymphocytes. They have a stable phenotype after withdrawal of the cytokines used in the *ex vivo* cultures.

The method of the invention affords immature dendritic cells and mature dendritic cells.

In the presence of GM-CSF and an interleukin, $CD83^{-}$ $CD14^{low}$ immature dendritic cells are obtained. These DC express HLA-DR, CD80 and CD86 as well as endocytic and phagocytic receptors, namely MR, CD36 and $\alpha v\beta 5$.

In addition, these immature DC are capable of phagocytosing apoptotic tumor cells by the phagocytosis of apoptotic monocytes. Stimulation of these immature DC with TNF-α plus GM-CSF and IL-4 for a further 2 days affords cells which correspond in phenotypic and functional terms to mature dendritic cells. These mature cells expressed CD83 and larger amounts of HLA-DR, CD80 and CD86 than GM/IL-4 immature DC.

They are capable of activating allogenic T lymphocytes with the same efficiency as mature DC obtained in the presence of FCS.

In addition, these mature DC also express endocytic receptors such as mannose receptors or type $\alpha v\beta 5$ and CD36 phagocytosis receptors. However, the capacity of these mature DC to endocytose dextran or phagocytose apoptotic tumor cells is lower than that of the immature DC from which they are derived.

The chemokine response of the DC obtained according to the invention was modulated in a similar manner to that of the DC obtained in medium containing FCS. In fact, the immature DC obtained according to the invention expressed CCR5 and did not respond to MIP-3β. Thus, after *in vivo* injection, these cells should be trapped preferentially in inflammatory sites where MIP-1α, MIP-1β or RANTES are produced (25). After treatment with TNF-α, the DC according to the invention lost the expression of CCR5 and acquired the capacity to respond to MIP-3β. It is plausible that a high proportion of these mature DC will be capable of being trapped in lymphatic node zones of T cells where MIP-3β is produced, and of initiating an efficient immune response.

Furthermore, it has been shown that PGE2, which is known to enhance the maturation of DC in FCS-free media (14,21), can play a role in the migration of DC. Under the culture conditions according to the invention, PGE2 did not greatly

modify the phenotype of the DC produced with GM/IL-4 and TNF, with the exception of an increase in the expression of CD83, and does not have a supplementary additive effect with TNF-α for T cell activation. However, PGE2 increased DC migration in response to MIP-3β. DC migration into the lymphoid organs can therefore be selective.

To induce the production of antitumor cytotoxic T cells, the DC must be capable of directing the differentiation of naive T cells towards the type 1 subset expressing IFN-γ and IL-2. IL-12 is the main cytokine involved in the polarization of TCD4+ cells towards Th1 cells. In the anti-EBV T cell response model, it was demonstrated that the expression of IL-10 by lymphoblastoid cell lines is associated with the emergence of type 2 CD8+ T cells (32) which produce IL-4 and IL-10 and are not cytotoxic (33). By contrast, type 1 CD8+ cells produce IFN-γ and IL-2 and are cytotoxic. Thus DC produced *in vitro* for purposes of antitumor vaccination must ideally produce IL-12 and not IL-10. IL-10 actually has an additional noxious effect on the maturation of DC. In fact, DC treated with IL-10 during the maturation phase induce an antigen-specific anergy in CD4+ and CD8+ T cells (25,34). The DC obtained according to the invention, which differ from those obtained in the presence of FCS (35,36), produced only small amounts of IL-12 but large amounts of IL-10 in response to CD40 ligation, which was consistent with studies showing that myeloid DC were capable of producing IL-10, particularly following stimulation by CD40 (37,38).

However, it was shown that the maturation of DC induced by TNF-α caused the induction of IL-12 production and a dramatic inhibition of IL-10 synthesis after activation by CD40. Thus mature DC according to the invention are capable of triggering the differentiation of naive T lymphocytes into type 1 T lymphocytes. Furthermore, the addition of PGE2 inhibited IL-10 production, but also IL-12 production mature obtained.

The invention further relates to a method of immunotherapeutic treatment which consists in taking mononuclear cells from a patient to be treated by cytapheresis after mobilization by chemotherapy and/or with a cell growth factor and optionally freezing/thawing, treating said cells by the method defined above and activating them during step 2) of said method (maturation step) with specific antigens using the normal procedures well known to those skilled in the art, for example by endocytosis, and then reinjecting the resulting DC into said patient.

Advantageously, these dendritic cells can be frozen after the maturation/

activation step by the normal techniques, without substantial modification of their properties.

The DC according to the invention are particularly suitable for allograft or autograft treatments.

The invention will be illustrated in greater detail by the Examples below, which are given without implying a limitation, and by the Figures below, in which:

- Figure 1 shows the effect of the maturation of DC on the endocytosis of FITC-dextran:

A) with DC obtained by culture on X-VIVO 15 medium, 2% HA, in the presence of GM-CSF and IL-4;

B) with DC obtained by culture on X-VIVO 15 medium, 2% HA, in the presence of GM-CSF, IL-4 and TNF-α;

C) with DC obtained by culture on X-VIVO 15 medium, 2% HA, in the presence of GM-CSF, IL-4 and PGE2.

The dotted curves correspond to an incubation time of the DC with FITC-dextran of 7 minutes, the solid curves to incubation for 15 minutes and the emboldened curves to incubation for 30 minutes; the fluorescence intensity is plotted on the abscissa and the number of events on the ordinate.

- Figure 2 shows the apoptosis of XG-1 cells by cycloheximide (CHX) by measurement of the fluorescence of the cells stained with Annexin-V FITC and with propidium iodide (PI).

- Figure 3 shows the phagocytosis of apoptotic tumor cells by immature DC and the absence of phagocytosis by mature DC.

- Figure 4 shows the effect of the maturation of DC on the expression of CCR5.

- Figure 5 shows the migration of mature DC and the non-migration of immature cells in response to (ELC)/MIP-3β.

- Figure 6 shows the activation of allogenic T cells by mature DC.

EXAMPLE 1: Production of dendritic cells (DC)

Apheresis cells (AC) were collected from four patients presenting with different cancers during the mobilization of hemopoietic precursors with cyclophosphamide and human granulocyte colony stimulating factor (G-CSF, filgrastim; NEUPOGEN, Amgen-Roche, Neuilly-sur-Seine, France). Each batch of AC collected was frozen in liquid nitrogen and then thawed and washed twice in the presence of a calcium and magnesium chelator. Each batch of cells was then placed in a cell culture flask containing X-VIVO 15 medium supplemented with 2% of human albumin (X-VIVO, 2% HA) and the cells were allowed to adhere to the surface of the culture flask for 2 h. The non-adherent cells were discarded and the adherent cells were cultivated in the presence of 100 ng/ml of GM-CSF (Leucomax, Sandoz, Basle, Switzerland) and 25 ng/ml of IL-4 (R&D Systems, Minneapolis, MN) for 7 days in X-VIVO 15 medium supplemented with 2% of HA. For comparison purposes, adherent cells were also cultivated in the presence of 100 ng/ml of GM-CSF and 25 ng/ml of IL-4, either in RPMI 1640 medium supplemented with 10% of FCS (reference medium), or in X-VIVO 15 medium alone, or in X-VIVO 15 medium supplemented with 5% of ABS, 5% of autologous serum or 5% of autologous plasma. In each case, fresh medium containing GM-CSF and IL-4 was added on days 2 and 4. After 5 days of culture, medium was added which contained GM-CSF and IL-4 with TNF-α (R&D Systems) at 20 ng/ml or TNF-α at 20 ng/ml and PGE2 (Sigma Chemical, St Louis, MO) at 1 µg/ml. After 48 h, the cells were collected and counted. The cell yield is given in Table I, where it is seen that the cell yield obtained in the presence of GM-CSF and IL-4 reached 12% with AB serum, 18% with autologous plasma, 22% with autologous serum and 16% with HA. X-VIVO 15 medium, 2% HA, supplemented with GM-CSF and IL-4 is the most efficient medium for obtaining clinical grade $CD14^{-/low}$ $CD83^{-}$ $HLA\text{-}DR^{++}$ immature DC expressing large amounts of CD80 and CD86.

As the results in Table I also show, AB serum, autologous plasma and autologous serum were less active than HA for obtaining mature DC *in vitro*.

Given that a 5-hour cytapheresis generally enables the recovery of 40 to 50 x 10^9 mononuclear cells, 6 to 8 x 10^9 DC could therefore be obtained reproducibly under the operating conditions of the invention. This amount of cells is sufficient for at least 6 vaccinations with 10^9 DC.

Cell culture on X-VIVO 15 medium alone was performed using the apheresis cells from 8 donors, mobilized with hemopoietic growth factor (G-CSF)

or with cyclophosphamide and hemopoietic growth factor.

For 5 of the 8 donors, cells cultivated in X-VIVO 15 medium in the presence of GM-CSF and IL-4 for 7 days had a viability of less than 65%, which did not allow analysis of their phenotype or functions, in contrast to cells taken from the same donors and cultivated in the presence of X-VIVO 15, 2% human albumin, GM-CSF and IL-4 for 7 days.

X-VIVO 15 medium alone does not allow the reproducible generation of immature dendritic cells. The addition of 2% of human albumin made it possible in all cases to generate perfectly viable, functional and irreversible dendritic cells.

EXAMPLE 2: Phenotypic analysis of DC by flow cytometry

To characterize the phenotype of the DC obtained according to Example 1, the percentage of cells expressing CD14, HLADR, CD83, CD80 and CD86 was determined by flow cytometry (FACS) using the following monoclonal antibodies: CD1a-PE, CD14-PE, CD36-FITC, CD80-PE, CD83-PE, HLA-DR-FITC (Immunotech, Marseille, France) and CCR5-PE, CD51/CD61-FITC, CD86-FITC, MR-PE (Pharmingen, San Diego, CA), and isotype-matched murine IgG antibodies (Immunotech).

The total phenotype of the DC obtained according to Example 1 is similar to that of the immature DC obtained by culture in RPMI medium in the presence of FCS (Table I). When using X-VIVO 15 medium supplemented with AB serum, autologous plasma or autologous serum, the percentage of $CD14^+$ cells was greatly increased (to 80% in X-VIVO 15, AB serum) and the resulting cells expressed a lower density of HLA class II and co-stimulatory molecules. On the other hand, in the method of the invention, i.e. with X-VIVO 15 medium, 2% HA, it was possible to obtain a high number of DC ($CD14^-$, $HLA\text{-}DR^{++}$, $CD80^{++}$, $CD86^{++}$) without the addition of xenogenic proteins, allogenic proteins, human antibodies or unidentified autologous tumoral antigens.

Similar results were obtained with AC taken from fifteen donors and cultivated under the operating conditions of the invention, i.e. in X-VIVO 15 medium supplemented with 2% of human albumin, in the presence of GM-CSF, IL-4 and TNF-α, with or without PGE2. These results are shown in Table II.

CD83, a specific marker for mature DC, could be detected after culture for 24 h in the presence of TNF-α and reached maximum expression in 48 h. The combination of PGE2 and TNF-α induced the expression of CD83 up to 83% of

cells, compared with 64% in the case of TNF-α alone (p = 0.007) (Table II). PGE2 also cooperated with TNF-α to upregulate CD80 and CD86 on the DC (Table I and Table II).

Another series of experiments was carried out under the same conditions as above, except that PGE2 without TNF-α was added on day 5.

The percentage of $CD14^+$ cells obtained in the presence of GM-CSF + IL-4 + PGE2 was greater than that obtained in the presence of GM-CSF and IL-4 alone, suggesting that when it is used without TNF-α, PGE2 induces the reversion of at least some immature DC to macrophage-type cells, even though GM-CSF and IL-4 were continually present in the culture medium.

Likewise, when immature DC obtained in X-VIVO 15, 2% HA, supplemented with GM-CSF and IL-4 were harvested on day 7, washed extensively and cultivated in cytokine-free culture medium for a further 3 days, they again adhered to the culture bag and expressed CD14. This is a case of reversion of these immature DC to macrophage-type cells. On the other hand, the cell morphology and cell phenotype of mature DC produced by adding TNF-α alone or TNF-α + PGE2 were not markedly affected after withdrawal of the cytokines, indicating that maturation took place irreversibly.

EXAMPLE 3: Endocytosis mediated by MR

Endocytosis at the cellular level was studied on DC obtained by the culture of AC mobilized by the treatment indicated in Example 1, in X-VIVO 15 medium, 2% HA, in the presence of GM-CSF and IL-4 for five days. On day 5, fresh medium was added which contained GM-CSF and IL-4, or GM-CSF, IL-4 and TNF-α, or GM-CSF, IL-4, TNF-α and PGE2. On day 7, the expression of MR, CD36, αvβ3 and αvβ5 was determined by the FACS method of analysis.

Expression of the MR marker was determined by the method described by Tarte et al. (27) using lysine-binding FITC-dextran, MW = 40,000 (Molecular Probes Inc., Eugene, OR). The immature and mature DC were collected on day 7 and incubated at 37°C for 7, 15 and 30 min or at 4°C for 30 min (background binding) with 1 mg/ml of FITC-dextran. The DC were then washed with cold PBS supplemented with 1% of FCS and 0.02 of NaN_3 and the fluorescence was analyzed with a FACScan apparatus.

The mean percentage of positive cells obtained by the culture of AC from six donors is indicated in Table III.

These results show that the immature DC obtained in X-VIVO 15, 2% HA, by culture for 7 days with GM-CSF and IL-4 expressed substantial amounts of MR and that this expression decreased significantly by more than 50% on maturation of the DC induced either by TNF-α alone ($p = 0.03$) or by TNF-α + PGE2 ($p = 0.03$).

The endocytosis of FITC-dextran by the mature cells obtained according to the invention with TNF-α or TNF-α + PGE2 decreased very appreciably compared with the immature DC, as shown in Figure 1.

EXAMPLE 4: Induction of apoptosis in malignant plasma cells

The phagocytic potential of the DC obtained by the method of the invention was tested using apoptotic tumor cells.

XG-1 is a multiple myeloma cell line whose characteristics have been described in detail by Zhang et al. (28). XG-1 cells (2.5×10^5/ml) were incubated with 4 µm/ml of cycloheximide (CHX) in RPMI 1640 medium, 10% FCS, supplemented with 3 ng/ml of IL-6 at 37°C. The kinetics of cell apoptosis were recorded using double staining with the dye known under the name Annexin-V FITC (Boehringer Mannheim, Meylan, France) and propidium iodide (PI) (Sigma). Initially the apoptotic cells were stained only by Annexin-V (Annexin-V^+/PI^-), whereas subsequently the necrotic cells also incorporated PI due to a loss of membrane integrity (Annexin-V^+/PI^+). After treatment with CHX, the tumor cells were washed three times in X-VIVO 15, 2% HA, before being co-cultivated with DC obtained by the procedure described in Example 1.

The results obtained are reported in Figure 2. These results show that, after 6 h of culture with 4 µm/ml of CHX, 60% of the XG-1 myeloma cells exhibited characteristics of early apoptotic cell death, i.e. binding of Annexin-V but non-incorporation of PI.

EXAMPLE 5: Phagocytosis of apoptotic cells

The phagocytosis of apoptic cells represents another mode of entry for antigens and plays a major role in the phenomenon of cross priming. Recently, several phagocytic receptors have been identified on DC obtained in the presence of human sera, and it has been shown that a monocyte conditioned medium (MCM), which leads to irreversible DC maturation, downregulates their expression (6).

Immature and mature DC were stained green using PKH67-GL (Sigma) and

were cultivated for 2 h to release the unbound dye. XG-1 cells were stained red using PKH26-GL (Sigma) according to the manufacturer's instructions, before being induced to undergo apoptosis with CHX for 6 to 8 h. Red-stained XG-1 cells were then co-cultivated with green-stained immature or mature DC in a ratio of 1:1 in X-VIVO 15, 2% HA, according to the protocol described by Albert et al. (6). After 90 min at 37°C, the green and red fluorescences were analyzed with a FACScan apparatus. In blocking experiments, XG-1 cells and DC were co-incubated at 4°C.

The CD36, αvβ3 and αvβ5 markers were determined by the monoclonal antibody labeling method and flow cytometry.

To stain αvβ5, the cells were first incubated with an αvβ5 primary mAb (Chemicon Int., Temecula, CA) and then with an FITC-conjugated anti-mouse Ig goat antibody (Immunotech). The analyses were performed with a FACScan apparatus (Becton Dickinson).

The data derived from one experiment representative of 3 are shown in Figure 3. More than a third of the immature DC engulfed apoptotic XG-1 after 90 min of co-culture. Only 10 to 12% of the immature DC were stained twice after co-culture with non-apoptotic XG-1 cells. The phagocytosis of tumor cells by immature DC was confirmed visually on stained cytokines from co-culture. Phagocytosis was completely blocked at low temperature (Figure 3). Induction of the maturation of DC produced a decrease in phagocytic activity. In fact, only 12% of the mature DC obtained after the addition of TNF-α internalized the apoptotic XG-1 after 90 min of co-culture (Figure 3). The same decrease in phagocytosis occurred with mature DC obtained with TNF-α + PGE2 (Figure 3).

Under the conditions of the invention, immature DC expressed large amounts of CD36 and αvβ5 integrin; by contrast, αvβ3 integrin was not detected, as shown by the results collated in Table III. These results also show that, in the presence of TNF-α, the expression of CD36 and αvβ5 was significantly reduced by more than half (p - 0.002) and by 20-35% (p = 0.03) respectively. With TNF-α, PGE2 did not have an additional effect in terms of reducing the expression of the phagocytic receptors.

EXAMPLE 6: Response of DC to chemokines

The operation was repeated with six different donors and the mean fluorescence intensity (MFI) was measured. The results obtained are shown in

~~Table 4.~~

1) detection of the CCR5 receptor

In this test, the anti-CCR5 monoclonal antibody (Pharmingen, San Diego, CA, USA) was used to detect the CCR5 receptor, which is a receptor for inflammatory chemokines.

The mature or immature DC obtained were incubated in X-VIVO 15 medium, 2% HA, with said labeled antibody and the expression of CCR5 was measured for one donor. The results are collated in Figures 4a, 4b and 4c.

The aim of this Example was to look for the presence of CCR5 on the immature DC and mature DC produced in X-VIVO 15 medium, 2% HA.

CCR5 was detectable on the immature DC produced in X-VIVO 15 medium, 2% HA, but its expression was significantly reduced by incubation for 48 h with TNF-α ($p = 0.03$). PGE2 did not induce a significant additional reduction (Figure 4) ($p = 0.25$).

2) detection of the CCR7 receptor

As no monoclonal antibody was available for measuring the expression of CCR7, the response of DC to MIP-3β was tested in a chemotaxis assay.

Immature and mature DC (2×10^5 cells) were introduced into 100 µl of RPMI, 1% HA, in the upper chamber of a cell separation device consisting of two cell culture chambers (a lower chamber and an upper chamber separated by a filter with 5 µm pores to allow passage of the migratory cells (Transwell device from Costar, Cambridge, MA)). 600 µl of ELC/MIP-3β, diluted to 100 ng/ml in the same medium, were introduced into the lower chamber. After incubation for 4 h at 37°C, the cells which had migrated into the lower chamber were collected and counted under a microscope. The results were expressed as the percentage of initial cells which had migrated into the lower chamber (percentage of migratory cells). The migration of DC originating from a donor in the absence and presence of MIP-3β is shown in Figure 5A and the results obtained with DC derived from AC taken from 6 donors with MIP-3β are collated in Figure 5B. Immature DC cultivated with GM-CSF and IL-4 did not respond to MIP-3β (mean percentage of migrated cells: 0.7%, $n = 6$). The addition of TNF-α on day 5 significantly increased the response of the DC ($p = 0.002$), the mean percentage of migratory cells being 14.2 ($n = 6$). PGE2 acted synergistically with TNF-α since 31 to 67% (mean: 48.8%, $n = 6$) of the DC matured with TNF-α + PGE2 migrated into the lower chamber of the Transwell device over an incubation period of 4 h at 37°C (p

= 0.002), compared with the DC matured with TNF-α. This was associated with a slight increase in the spontaneous migration of the DC (a mean of 7% of the initial DC were found in the lower chamber in the absence of MIP-3β).

EXAMPLE 7: Analysis of the cytokines

The immature and mature DC obtained after 7 days of culture in X-VIVO 15, 2% HA, were harvested, washed and plated at a rate of 4 x 10^5/ml in RPMI 1640, 5% FCS, with or without L cells transfected by 10^5/ml of CD40L (supplied by Doctor Sem Saeland, Schering-Plough, Dardilly, France). At the indicated time, recombinant human IFN-γ (1000 U/ml, R&D Systems) was added. The supernatants were collected 24 h to 30 h after stimulation and stored at -70°C. The amounts of IL-10 and p70 IL-12 were measured by ELISA according to the manufacturer's protocol (R&D Systems).

A8 The immature DC obtained with GM-CSF/IL-4 did not produce p70 IL-12, but did produce very large amounts of IL-10 after triggering by CD40 (Table 5). The addition of IFN-γ together with stimulation by CD40 caused a 30-fold decrease in the production of IL-10 by immature DC activated by CD40. Induction of the maturation of DC with TNF-α caused a dramatic decrease in the production of IL-10 induced by CD40 (10-fold mean reduction), in association with induction of the expression of IL-12. The addition of IFN-γ again inhibited the production of IL-10 by mature DC. This is consistent with previous reports showing that IFN-γ could be a co-factor for the production of IL-12 induced by CD40 (29,30). However, for the test sample from the other three patients, IFN-γ reduced the production of IL-12 by DC obtained in the presence of GM-CSF/IL-4 and TNF-α. Finally, induction of a totally mature DC with TNF-α and PGE2 caused a reduced production of IL-10 and IL-12 after stimulation by CD40, compared with TNF-α ~~alone.~~

EXAMPLE 8: Allogenic mixed lymphocyte reaction (MLR)

A9 Non-activated T lymphocytes (HLA DR^-) were purified from healthy volunteers' peripheral blood by two negative selection cycles using microbeads coated with CD14 and CD19 (Dynal, Oslo, Norway), followed by a cocktail of CD16, CD65 and HLA-DR mAbs (Immunotech) and anti-mouse Ig goat microbeads (Dynal). The purity of the $CD3^+$ T cells was greater than 97%. Increasing numbers of DC treated with mitomycin (50 μg/ml) were added to 1.5 x

10^5 allogenic T cells in 200 µl of RPMI, 5% ABS. After 5 days of culture, the T cell proliferation was measured by the incorporation of tritiated thymidine (1 µCi/well) over the last 12 hours. The results were expressed as the mean costs per minute (cpm) ± standard deviation, determined in sextuplet culture wells.

The results in Figure 6 show that maturation of the DC obtained in X-VIVO 15, 2% HA, converted them to allogenic T cell stimulators of the same potency as the mature DC produced in RPMI, 10% FCS. TNF-α alone gave the same results as the association of TNF-α and PGE2.

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Table I

Phenotypic analysis of DC

Medium	Cytokines	Yield (%)	Viability (%)	Mean percentages of positive cells (MFI)				
				CD14	HLA-DR	CD83	CD80	CD86
RPMI-FCS	GM/IL-4	9	93	10 (32)	100 (180)	3	80 (72)	82 (53)
	GM/IL-4/TNF	10	94	4	100 (465)	86 (47)	98 (120)	90 (126)
	GM/IL-4/TNF/PGE2	10	95	3	100 (417)	94 (65)	98 (190)	100 (188)
XV-AB serum	GM/IL-4	12	92	80 (52)	100 (115)	0	77 (32)	90 (22)
	GM/IL-4/TNF	14	90	25 (69)	100 (224)	25 (40)	95 (60)	85 (50)
	GM/IL-4/TNF/PGE2	13	86	40 (50)	100 (173)	52 (80)	90 (60)	100 (95)
XV-autologous plasma	GM/IL-4	18	90	40 (35)	100 (90)	0	25 (30)	90 (65)
	GM/IL-4/TNF	19	95	20 (29)	100 (125)	20 (55)	35 (42)	90 (110)
	GM/IL-4/TNF/PGE2	23	92	12 (60)	100 (145)	80 (65)	80 (58)	110 (160)
XV-autologous serum	GM/IL-4	22	89	50 (35)	100 (95)	9 (12)	28 (26)	85 (68)
	GM/IL-4/TNF	25	90	26 (66)	100 (120)	17 (25)	39 (33)	90 (92)
	GM/IL-4/TNF/PGE2	23	93	40 (70)	100 (115)	59 (66)	72 (68)	95 (150)
XV-HA	GM/IL-4	16	89	21 (13)	100 (172)	0	82 (46)	85 (80)
	GM/IL-4/TNF	16	94	5	100 (270)	55 (43)	100 (110)	95 (97)
	GM/IL-4/TNF/PGE2	20	90	3	100 (251)	85 (66)	100 (136)	100 (133)

XV-HA = X-VIVO 15 medium, 2 % human albumin

GM = GM-CSF factor

Table II

Phenolytic analysis of DC obtained by culture in XV-HA medium supplemented with GM-CSF, IL-4 and TNF-α, with or without PGE2

Cytokines	CD14	HLA-DR	CD83	CD80	CD86
GM/IL-4/TNF	2.6 ± 8.2	100	64 ± 19	100	100
Mean %*** ± SD	-	299 ± 183	44 ± 7	140 ± 49	251 ± 211
GM/IL-4/TNF/PGE2	1.5 ± 4	100	83 ± 12**	100	100
Mean %*** ± SD	-	265 ± 124	61 ± 25*	190 ± 85	345 ± 271

* $p < 0.01$ by comparison with cells cultivated with GM/IL-4/TNF

** $p < 0.05$ by comparison with cells cultivated with GM/IL-4/TNF

*** mean % = mean % of positive cells

GM = GM-CSF factor

Table III

Profile of DC receptors

Culture conditions	Mean % of positive cells (MFI)			
	MR	CD36	αvb3	αvb5
XV-HA GM/IL-4	98 (233)	88 (89)	0	87 (43)
XV-HA GM/IL-4/TNF	80 (95)*	37 (47)**	0	68 (32)*
XV-HA GM/IL-4/TNF/PGE2	74 (91)*	25 (33)**	0	58 (36)*

XV-HA = X-VIVO 15 medium, 2% HA

* $p < 0.01$ by comparison with cells cultivated with GM/IL-4

** $p < 0.05$ by comparison with cells cultivated with GM/IL-4

Table IV: CCR5

Culture conditions	GM+IL-4	GM+IL-4+TNF	GM+IL-4+TNF+PGE2
Donor 1	8	4	3
Donor 2	12	6	5
Donor 3	14	6	5
Donor 4	10	5	5
Donor 5	10	4	4
Donor 6	11	6	6
MFI	10.8	5.2	4.7
	p = 0.03		p = 0.25

Table V

Production of cytokines by DC

Culture conditions	Production of cytokines (pg/ml)					
	without stimulation		stimulation with CD40		stimulation with CD40 + IFN-γ	
	IL-10	IL-12	IL-10	IL-12	IL-10	IL-12
XV-HA GM/IL-4	25 ± 9	0	1619 ± 529	5.5 ± 6.3	50 ± 57	158 ± 274
	(16-35)		(1105-2360)	(0-11)	(14-115)	(0-476)
XV-HA GM/IL-4/TNF	0	0	137 ± 104	84 ± 23	7 ± 7	299 ± 518
			(62-285)	(55-105)	(0-21)	(0-898)
XV-HA GM/IL-4/TNF/PGE2	0	0	64 ± 61	7.2 ± 8.8	0	16 ± 29
			(0-145)	(0-18)		(0-50)